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Kv4.3 expression abrogates and reverses norepinephrine-induced myocyte hypertrophy by CaMKII inhibition

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Abstract

Background: Down-regulation of Kv4.3 protein is a general feature of cardiac hypertrophy. Based on our recent studies, we propose that Kv4.3 reduction may be a hypertrophic stimulator.

Objective: We tested whether Kv4.3 expression can prevent or reverse cardiac hypertrophy induced by norepinephrine (NE).

Methods and Results: Incubation of 20 μM NE in cultured neonatal rat ventricular myocytes (NRVMs) for 48 h and 96 h induced myocyte hypertrophy in a time-dependent manner, characterized by progressive increase in cell size, protein/DNA ratio, ANP and BNP, along with an progressive increase in the activity of CaMKII and calcineurin and reduction of Kv4.3 mRNA and proteins. Interestingly, PKA-dependent phosphorylation of phospholamban (PLB) at Ser16 was increased at 48h but reduced to the basal level at 96h NE incubation. CaMKII inhibitors KN93 and AIP blunted NE-induced hypertrophic response and caused regression of hypertrophy, which is associated with a reduction of CaMKII activity and calcineurin expression. Kv4.3 expression completely suppressed the development of NE-induced hypertrophy and led to a regression in the hypertrophic myocytes. These effects were accompanied by a reduction in CaMKII autophosphorylation, PLB phosphorylation at Thr-17 without changing PLB phosphorylation at Ser-16. NFATc3 was also reduced by Kv4.3 expression.

Conclusions: Our results demonstrated that Kv4.3 reduction is an important mediator in cardiac hypertrophy development via excessive CaMKII activation and that Kv4.3 expression is likely a potential therapeutic strategy for prevention and reversion of adrenergic stress-induced cardiac hypertrophy.

Keywords

I_{to} channel; Kv4.3; hypertrophy; calcineurin; CaMKII; myocytes

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CONFLICT OF INTEREST: none declared.

INTRODUCTION

Cardiac CaMKII is activated by calcified calmodulin (Ca^{2+}/CaM), a process initially triggered by intracellular Ca^{2+}. Once activated, CaMKII becomes autophosphorylated and independent of its activators[1]. There is growing evidence implicating CaMKII in the development of cardiac hypertrophy and failure[2]. Studies performed in animal models, *in vitro* systems and human subjects suggest that CaMKII is a key mediator for Ca^{2+} handling and a trigger for transcriptional response of hypertrophic gene program[3–5]. CaMKII has been suggested to modulate gene expression *via* different transcription factors, including activation protein 1 (AP-1), CAAT-enhancer binding protein, activating transcription factor (ATF-1), serum response factor (SRF), cAMP-response element binding protein (CREB), and myocytes enhancer factor 2 (MEF2)[6]. Recent studies showed that histone deacetylases (HDACs) serve as functional links between CaMKII and MEF2[7] HDAC4 is phosphorylated by CaMKII. Phosphorylated HDAC4 translocates into the cytoplasm by 14-3-3 protein, resulting in derepression of the MEF2 gene, leading to hypertrophic growth[8]. In addition, it has been demonstrated that the CaMKII/HDAC pathway also synergizes with the calcineurin/NFAT pathway to induce profound cardiac hypertrophy[9].

Prominent transient outward K^+ current (I_{to}) has been recorded in ventricular myocytes isolated from the hearts of many species, including mice, rats, rabbits, cows, cats, dogs, ferrets and humans[2]. Depending on the species and regions in heart, there are at least two distinct I_{to} phenotypes, namely $I_{to,f}$ and $I_{to,s}$. $I_{to,f}$ is mediated by Kv4.3 and/or Kv4.2 channels, whereas $I_{to,s}$ is mediated by Kv1.4 channels. I_{to} reduction is a well-known feature of hypertrophic ventricular myocytes. Reductions in Kv4.3/Kv4.2 protein expression, but not Kv1.4, have been consistently linked to the diminished I_{to} densities observed in cardiac hypertrophy[10].

In our recent study, we uncovered a mechanism that Kv4.3, by binding to the CaM binding sites on the inactive CaMKII, prevents Ca^{2+}-induced CaMKII activation[11]. As extension of this work, here, we tested the role of Kv4.3 expression in norepinephrine (NE)-induced hypertrophic response in neonatal rat ventricular myocytes.

METHODS

Antibodies, reagents and adenoviruses

Primary antibodies used in this study include rabbit polyclonal and mouse monoclonal anti-CaMKII antibodies (M-176 and G-1), goat polyclonal anti-PLB antibody, rabbit polyclonal anti-calcineurin antibody, rabbit polyclonal anti-NFATc3 antibody, goat polyclonal anti-ANP antibody (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-protein kinase A antibody (Upstate Biotechnology, Lake Placid, NY), polyclonal anti-phospho-CaMKII (Thr287) antibody (Cell Signaling solutions, Lake Placid, NY), rabbit polyclonal anti-P-Thr17PLB and P-Ser16PLB antibodies (Badrilla, Leeds, UK), and mouse monoclonal anti-GAPDH antibody (Millipore Corporation). Secondary antibodies used for Western blot were donkey anti-goat IgG-HRP, goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology).

Other reagents used in this study include KN93 and AIP (Calbiochem), vitamin C (ascorbic acid), cyclosporin A and norepinephrine (Sigma). Recombinant adenoviruses harboring GFP-β-gal, GFP-Kv4.3, and GFP-Kv4.2 used for neonatal ventricular myocyte infection were kind gifts from Dr. Djamel Lebeche (Cardiovascular Research Center, Mount Sinai School of Medicine, USA).

Neonatal rat ventricular myocyte isolation and adenoviral transfection

Neonatal rat ventricular myocytes (NRVMs) were isolated from postnatal day 1 Sprague-Dawley rats as described previously[12]. Cells were pre-plated in petri dishes for 2h to selectively enrich cardiomyocytes and then plated in cell culture dishes with a density of 300 cells/mm^2 and cultured for 24 h in DMEM/M199 (3:1) containing 5% FBS and 10% horse serum in the presence of 100 mM bromodeoxyuridine (BrdU; Sigma). The culture medium was then replaced with serum-free DMEM/M199. For hypertrophic response induction, norepinephrine (NE, 20μM) was added for 48 and 96 h. All inhibitors (1μM KN93, 10μM AIP and 5μM cyclosporine A) were added 2h prior to NE addition, respectively.

For adenovirus-mediated gene transfection, myocytes were incubated with Ad-β-gal or Ad-Kv4.3 adenovirus at a multiplicity of infection (MOI) of 200. For experiments testing the reversibility of hypertrophy, NE was applied to the cultured neonatal myocytes for 48h to induce hypertrophy, kinase inhibitors or adenoviruses (Ad-β-gal or Ad-Kv4.3) were then added to the culture medium for another 48h.

Measurement of cell surface area

Cell images were obtained using phase-contrast fluorescence microscope with a 10X objective lens. Image processing software NIH Image (National Institutes of Health, Bethesda, MD, USA) was used to determine cell size. Cell surface area was estimated by averaging at least 150 myocytes for each group.

Measurement of protein/DNA ratio

Myocytes were lysed in 200μl of lysis buffer containing 1% Triton X-100 and 10 ng/ml of ribonuclease A for 30min on ice. For protein assays, a 5μl aliquot was mixed with BCA protein assay reagent (Thermo Scientific, MA, USA), and measurements were conducted by microtiter plate reader according to the manufacturer's instructions. Similarly, another 5μl aliquot was mixed with Pico-Green reagent (Molecular Probes, Eugene, OR, USA), and DNA concentrations were measured against a standard curve, according to the manufacturer's instructions.

RNA analysis

Total RNAs were isolated from myocytes using TRIzol Reagent (Invitrogen). Extracted RNA was subsequently purified using PureLink™ purification Kit (Invitrogen). DNA was removed by DNase I treatment before cDNA synthesis. RNA concentration was determined using absorbance 260nm spectroscopy. For reverse transcriptional polymerase chain reaction (RT-PCR), the cDNAs were synthesized from total RNA (1μg) using SuperScript® III First-Strand Synthesis System (Invitrogen). PCR mixture contained 1X of PCR buffer, 200μM of each dNTP, 1.5mM $MgCl_2$, 200μM of sense and antisense primers, 5μ1 of cDNA and 1 unit

of Platinum Taq polymerase (Invitrogen). PCR primers derived from rat sequences were: CaMKII (sense 5'-TGACACCTGAAGCCAAAGACCTCA-3'; antisense 5'-TACAGTCTCCTGCCTGTGCAT CAT-3'); calcineurin (sense 5'-CCACAGGGATGTT GCCTAGTG-3'; antisense 5'-GTC CCGTGGTTCTCAGTGGTA-3'); Kv4.3 (sense 5'-AG CTGTGCCTCAGAACTAGGCTTT −3'; antisense 5' -TACCAGAAAGACGCAGGGATGCTT-3'); BNP (sense 5'-TCTCAA AGGACCAAGGCCCTACAA-3'; antisense 5'-CTTCTGC CCAAAGCAGCTTGAACT-3'); GAPDH (sense 5'-TGACTCTACCCACGGCAAGTTCAA- 3'; antisense 5'-ACGACATAC TCAGCACCAGCATCA-3'). The expression level of GAPDH was used as internal control. PCR products were separated by electrophoresis. Bands were detected at expected size for CaMKII (142bp), Calcineurin (242bp), Kv4.3 (134bp), BNP (131bp) and GAPDH (141bp) and analyzed using NIH software.

SDS-PAGE and immunoblotting

Proteins were electrophoresed on 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membrane (PVDF). Blots were blocked in 10 ml TBSTM (Tris-buffered saline with 0.1 % Tween 20 containing 5% (w/v) nonfat dry milk powder) for 1h at room temperature and then incubated in TBSTM containing 1:1000 diluted anti-CaMKII antibody for 12–16 h at 4°C. The blots were washed 3 times with TBST and then probed with 1:10,000 diluted goat anti-rabbit antibody conjugated-horseradish peroxidase (HRP) in TBSTM for 1 h at room temperature. Expression of other proteins was detected using specific antibodies as indicated. Protein bands were detected by incubating blots with SuperSignal West Dura Extended Duration Substrate ECL, (Pierce) and visualized using X-film developer (Konica). Anti-GAPDH antibody was used to normalize protein loading in each lane. The immunoreactive band intensity was quantified using the Image J software (NIH) and results were normalized to the respective levels of GAPDH.

Statistical Analysis

Data were reported as mean±SEM and analyzed by *t* test or one-way ANOVA (using Sigmastat for Windows V3.5). Mann-Whitney Rank Sum tests were performed if tests for normality or equal variance failed. $p<0.05$ is regarded as significant.

Use of Vertebrate Animals

All experiments on live vertebrates were performed in accordance with the protocols approved by the institution's Animal Care and Use Committee.

RESULTS

Time course of NE-induced hypertrophy in cultured NRVMs

Cardiac hypertrophy is a progressive process. To determine the time course of NE-induced hypertrophy in NRVMs, isolated myocytes were cultured in the culture medium containing 20μM NE for 48 to 96h. Cell images (Figure 1A) showed a significant increase in the size of myocytes after exposure to NE for 48 h, and cell size was further increased up to 96h incubation with NE, compared to the controls (cultured myocytes without exposure to NE). Cell surface area was increased by approximately 60% and 110% after NE treatment for 48

and 96h, respectively (Figure 1B). In line with the increase in cell size, protein/DNA ratio (a hypertrophic index) was continuously increasing during NE incubation for 48 - 96h (Figure 1C). NE-induced alterations of protein expression in neonatal myocytes were determined by Western blot analysis using specific antibodies (Figure 1 D-K). The results showed a progressive increase in the level of CaMKII autophosphorylation and CaMKII-dependent phospholamban (PLB) phosphorylation at Thr-17 with unchanged total proteins, indicating an increased overall and membrane-localized CaMKII activation. Unlike CaMKII, calcineurin level was increased at 48h NE treatment but reduced to the control level at 96h treatment. In addition, PKA-dependent PLB phosphorylation at Ser-16 was increased at the 48h but remarkably decreased at 96h NE treatment without change in PKA protein level. These results suggest that NE-induced hypertrophy in NRVMs involves activation of CaMKII, calcineurin and PKA, but the contribution of PKA and calcineurin declines with time after 48h NE treatment. For the sustained catecholaminergic stimulation (96h), CaMKII is the most important hypertrophic mediator. This is consistent with the signaling switch from PKA to CaMKII under persistent adrenergic stimulation[13].

Inhibition of CaMKII prohibits NE-induced hypertrophy in NRVMs

To dissect the role of CaMKII in the NE-induced hypertrophy, specific inhibitors of CaMKII were used. After 24 h serum-free culture, myocytes were treated with 1 μM KN93 (an allosteric inhibitor of CaMKII) for 2 h and then incubated with NE for 48 h. Results showed that KN93 effectively abrogated NE-induced increase in cell size and protein/DNA ratio (Figure 2 A-C). Western blot analysis showed that autophosphorylated CaMKII (p-CaMKII) and CaMKII-dependent PLB phosphorylation at Thr-17 (p-Thr17-PLB) were significantly decreased by KN93 treatment, indicating successful inhibition of CaMKII activity. In agreement with blunted hypertrophic response, atrial natriuretic peptide (ANP), a marker of myocardial hypertrophy[14,15], was increased by NE treatment but this increase was largely attenuated by KN93 (Figure 2 D-E). These results suggest that CaMKII is a key mediator for NE-induced hypertrophy. To further demonstrate this, we employed another CaMKII specific inhibitor, cell membrane permeable AIP (myristoylated AIP). This is a substrate-based inhibitor that directly binds to the catalytic domain. Similar to KN93, 10 μM AIP effectively eliminated NE-induced hypertrophic response (Figure 2 F-G).

NE-induced hypertrophy was reversible by inhibition of CaMKII

We showed that CaMKII autophosphorylation level was increased continuously in the entire course of hypertrophic process. One would question whether this hypertrophic response is reversible by inhibiting CaMKII. To test this, myocyte hypertrophy was first induced by NE incubation for 48 h, and then exposed to AIP for another 48 h. Cell images, surface area and protein/DNA ratio analysis showed that NE-induced hypertrophy was indeed reversed by treating the hypertrophied myocytes with AIP (Figure 3 A and B). Western blot results showed that the increased levels of p-CaMKII, p-Thr17PLB and ANP in hypertrophied myocytes were significantly reduced after 48 h treatment with AIP (Figure 3 C and D). The ANP, a hypertrophic biomarker, was reduced to the control level (Figure 3D).

Kv4.3 expression blocks NE-induced hypertrophy via CaMKII inhibition

It is known that reduction of I_{to} channel expression is a general feature of ventricular myocytes in hypertrophied heart[16]. To prevent this electrical remodeling, we expressed Kv4.3 in NRVMs using Ad-Kv4.3 transfection as we reported recently[11]. In line with our previous results, we observed a high efficiency for both Ad-Kv4.3 and Ad-β-gal transfection in NRVMs (Figure 4A). In addition, Kv4.3 proteins were significantly increased in the lysate of NRVMs transfected with Ad-Kv4.3 compared with those from untreated NRVMs and NRVMs transfected with Ad-β-gal. As expected, NRVMs transfected with Ad-Kv4.3 showed the lowest level of autophosphorylated CaMKII (Figure 4B). To test the inhibitory effect of Kv4.3 expression on NE-induced hypertrophy, 20 μM NE was added in culture medium together with Ad-Kv4.3 (at 200 MOI) for 48 h, while myocytes incubated with NE and Ad-β-gal (at 200 MOI) was used as control. Fluorescence cell images showed that Kv4.3 expression prohibited the NE- induced cell hypertrophy (compared with cells transfected with Ad-β-gal, Figure 4A). In the presence of NE for 48 h, cell surface area and protein/DNA ratio were significantly increased in myocytes transfected with Ad-β-gal but unchanged in myocytes transfected with Ad-Kv4.3 (Figure 4C). An increase in CaMKII and calcineurin mRNA levels was observed in myocytes transfected with Ad-β-gal but not in myocytes transfected with Ad-Kv4.3. In addition, BNP mRNA level showed 2-fold increase in myocytes transfected with Ad-β-gal + NE (compared with myocytes treated with Ad-β-gal only) but unchanged in myocytes transfected with Ad-Kv4.3 + NE (Figure 4D-E). In parallel with these hypertrophic changes, Kv4.3 expression significantly reduced CaMKII and calcineurin activity (Figure 4 F-G). These results suggest that prevention of Kv4.3 down-regulation could effectively abrogate the NE-induced hypertrophy.

Reversal of the NE-induced hypertrophy by Kv4.3 expression

It is known that in the developed cardiac hypertrophy, Kv4.3 is down-regulated. Here, we tested whether cardiac hypertrophy is reversible by reversion of Kv4.3 expression. Cultured NRVMs were exposed to NE for 48 h to induce hypertrophy and followed by transfection with Ad-β-gal or Ad-Kv4.3 for additional 48 h. Gene transfection efficiency was monitored by detecting GFP expression under fluorescence microscope. 20 μM NE induced significant cell growths at 48 h culture, and myocytes transfected with Ad-β-gal experienced further enlargement during additional 48 h culture with NE. However, in Ad-Kv4.3 transfected myocytes, the enlarged cell size was reversed to the normal level after Ad-Kv4.3 transfection for 48 h (Figure 5 A). Statistical analysis showed that Kv4.3 overexpression totally reversed cell surface area and protein/DNA ratio (Figure 5 B). RT-PCR analysis demonstrated that sustained NE treatment for 96 h significantly increased CaMKII and calcineurin mRNA levels. Meanwhile, Kv4.3 mRNA level was dramatically decreased (by 50%) (Figure 5 C & D). Western blot results show that Ad-Kv4.3 transfection significantly reduced CaMKII autophosphorylation, CaMKII-dependent PLB phosphorylation at Thr17, and protein expression of NFATc3 and ANP (Figure 5E and F). These results demonstrated that reversion of the down-regulated v4.3 expression in hypertrophic myocytes can effectively reverse the NE-induced hypertrophy by inhibition of CaMKII and calcineurin activity.

DISCUSSION

Reduction of I_{to} density has been linked to altered AP profiles, especially the slowed early repolarization (which facilitates Ca^{2+} influx during action potential duration)[17], and this event occurs in the early stage of cardiac hypertrophy, indicating a possible role of I_{to} remodeling in the hypertrophic process[18]. Interestingly, some studies reported unexpected findings that inhibition of I_{to} results in cardiac hypertrophy. Wickenden *et al*[19] reported the first finding that cardiac-specific over-expression of a dominant-negative Kv4.2 in mice caused dilated cardiomyopathy and HF, in addition to the prolongation of APD. In a later study, Kassiri *et al*[20] reported that reduction of $I_{to,f}$ in cultured NRVMs caused hypertrophy *via* a calcineurin-dependent pathway. First, blocking of I_{tof} by heteropodatoxin, known as a specific blocker of Kv4.2 channel (without any effect on Kv1.4 channel), in cultured myocytes reduced $I_{to,f}$ density by ≈ 50%, increased cell capacitance by 30%, and increased protein synthesis by 23%. Second, over-expression of dominant-negative Kv4.2 channel subunits, selectively abolished $I_{to,f}$ and caused more than 2-fold prolongation of APD_{90} and a 47% increase in cell capacitance. However, these changes were prohibited by simultaneous over-expression of wild-type Kv4.2 channel subunits. In contrast, elimination of Kv1.4 channel current by over-expression of a dominant-negative transgene did not alter cell size or growth[20]. Shortly, Zobel *et al*[22]. reported that over-expression of Kv4.2 channels using adenovirus prevented the prolongation of APDs as well as the increases in Ca^{2+} influx and Ca^{2+} -transient amplitude induced by phenylephrine (PE). Thereafter, Lebeche *et al.* reported consistent data that over-expression of I_{to} channels Kv4.3 *in vivo* blunts the hypertrophic response in rats subjected to aortic stenosis[16]. Taken together, I_{to} channel α-subunits appear to play a crucial role in preventing cardiac hypertrophy. All of these studies reported a possible link between cardiac hypertrophy and APD prolongation from the Kv channel reduction and indicated the involvement of calcineurin activation. However, none of these studies have determined the time course of the hypertrophic development and the underlying signaling switch. Our results indicated an interplay of PKA, calcineurin and CaMKII activation in the process of hypertrophy development triggered by NE. We demonstrated that all these pathways were activated at the early stage of hypertrophy but the role of PKA and calcineurin declined with time while CaMKII activation persists. On the other hand, CaMKII inhibition itself can effectively suppress and reverse cardiac hypertrophy, indicating that the PKA and calcineurin activation play only a minor role, if any, in the NE-induced hypertrophy.

The reduction of I_{to} has been considered as a secondary change to cardiac hypertrophy for decades. However, we recently found that I_{to} channel subunits Kv4.3 binds to CaMKII, forming a dynamic molecular complex (Kv4.3-CaMKII units) in ventricular myocytes, which plays an important role in direct regulating CaMKII activity[11]. Kv4.3 knock-down significantly increases CaMKII activity, and Kv4.3 overexpression inhibits CaMKII activity. In the current study, we observed a significant reduction in Kv4.3 levels in the NE-induced hypertrophied neonatal ventricular myocytes at 48 h NE treatment with a further decrease during the hypertrophy development. Overexpression of Kv4.3 prevented the NE-induced CaMKII activation and totally abrogated the hypertrophic response. These results indicate

that Kv4.3 down-regulation may not simply be a disease-related secondary change. Instead, it serves as an important hypertrophic mediator.

However, Niwa *et al.* reported that Kv4.3 ablation in mouse heart did not produce cardiac hypertrophy[23] Several conditions need to be considered in this regard. For instance, CaMKII activity is not high at basic condition, where the PKA signaling plays a predominant role[2]. Therefore, deletion of Kv4.3 may not induce significant increase in CaMKII activity. As a result, cardiac hypertrophy was not observed in these mice. Under the condition of persistent cardiac stress, PKA signaling is desensitized due to the down-regulation of β1. In contrast, CaMKII signaling becomes a major player herein. Our studies showed that Kv4.3 is an important mechanism to prevent the membrane-localized CaMKII activation under stressed condition (*e.g.* the increased Ca^{2+} load)[11]. The membrane-localized CaMKII is more important than the general CaMKII because it directly phosphorylates membrane calcium channels and SR calcium regulatory proteins, by which amplifies the effect of stress signaling on $[Ca^{2+}]i$ mishandling. In other words, Kv4.3 plays a role in preventing hypertrophy at stressed condition although it may not be important for the normal heart. This may explain why Kv4.3 overexpression can prevent NE-induced hypertrophy.

Although the down-regulation of Kv4.3 is a general feature of cardiac hypertrophy that happens at the early stage, our data indicate that this process persists during the hypertrophy development. We have demonstrated that Kv4.3 expression not only abrogates hypertrophic response at the early phase but also reverses the hypertrophic remodeling in the developed hypertrophic myocytes. The underlying mechanisms involve the inhibition of the membrane-localized and the general CaMKII that derives from the direct Kv4.3 binding and APD shortening as we reported recently[2].

In consistent with the reversibility of cardiac hypertrophy in neonatal ventricular myocytes by Kv4.3-mediated CaMKII inhibition, a recent study[24] reported an interesting result that hemodynamic unloading in a pressure-overload heart failure mouse model resulted in normalization of LV structure, including normalization of the LV volumes, LV mass, and myocyte hypertrophy, accompanied by up to 80% normalization of the heart failure genes at 4 weeks after aortic debanding, although the underlying mechanisms were unclear. According to our results, we conclude that CaMKII inhibition is never too late to prevent or reverse cardiac hypertrophy.

Perspective

Pathological cardiac hypertrophy is a structural remodeling contributing to the diastolic dysfunction and sudden cardiac death. In the condition of persistent stress, it can progress to cardiac dilation and failure. A negative modulator or suppressor for the hypertrophic response is clinically important. In this study, we demonstrated that Kv4.3 down-regulation is not a simple secondary change to the pathology of cardiac hypertrophy. It is actually a hypertrophic mediator by triggering CaMKII activation. Expression of Kv4.3 not only prohibits but also reverses NE-induced cardiac hypertrophy, pointing to Kv4.3 as an intrinsic suppressor for the hypertrophic response induced by sustained catecholaminergic stimulation. Based on the notion that Kv4.3 down-regulation is a consistent finding in the structural heart diseases in both human and murine heart[2], our findings suggest that Kv4.3

gene transfection is likely a potent clinical therapeutic strategy for prohibiting and reversing cardiac hypertrophy.

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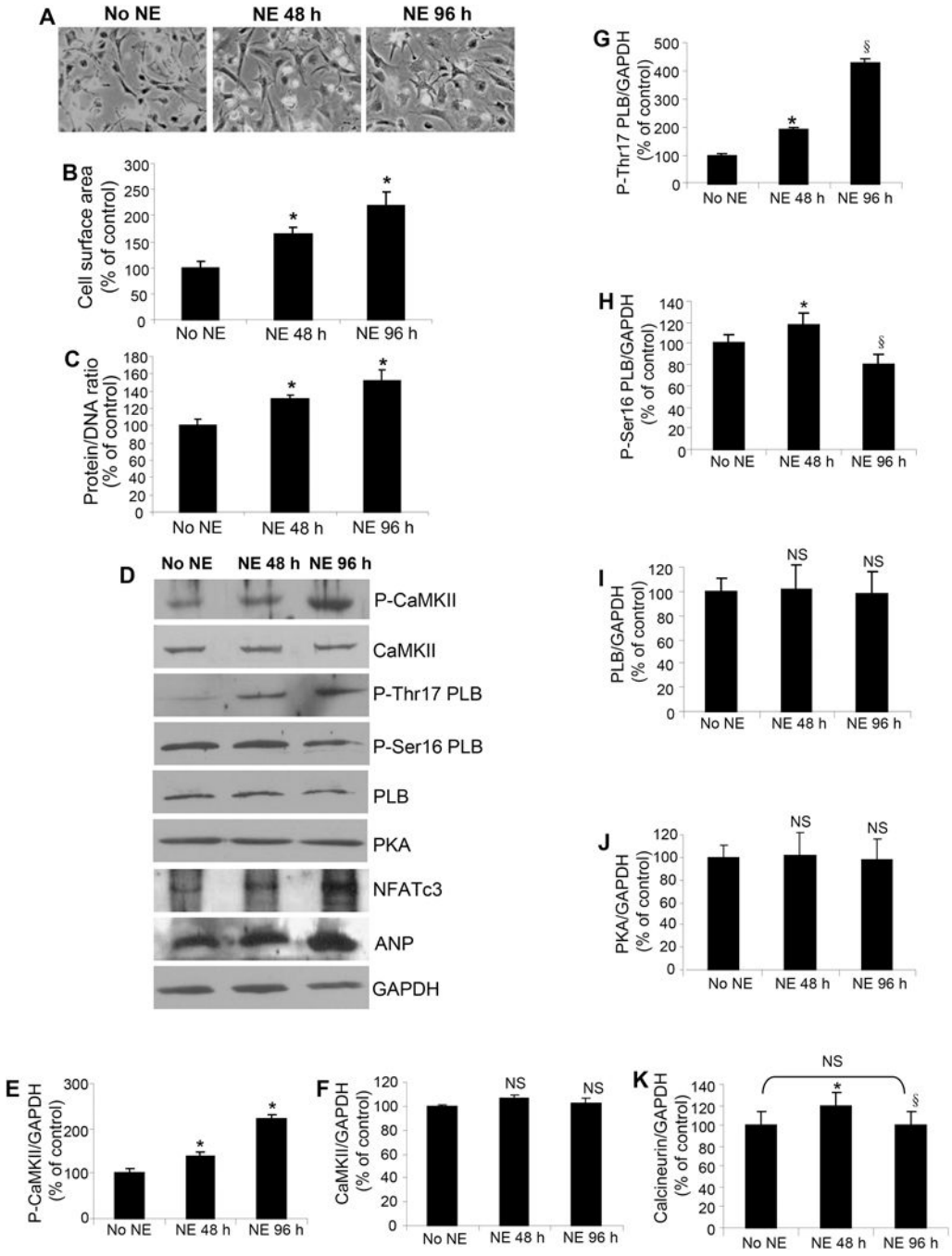

Figure 1. Time course of NE-induced hypertrophy in cultured NRVMs.

Cultured NRVMs were incubated with 20 μM NE for 48 and 96 h at 37 °C. **A:** Cell images showing increase in cell size with time. **B & C:** Statistical analysis of cell surface area and protein/DNA ratio in NRVMs incubated with NE for 48 and 96 h. Statistical data were mean values from 3 independent experiments. Vertical bars represent S.E.M. **D & K:** Western blot data of relative proteins. Each bar graph shows normalized protein expression levels from 3 independent experiments. Vertical bars represent S.E.M. *: $p < 0.05$, compared to control (No NE); §: $p < 0.05$, compared to NE 48 h. NS denotes no significant difference.

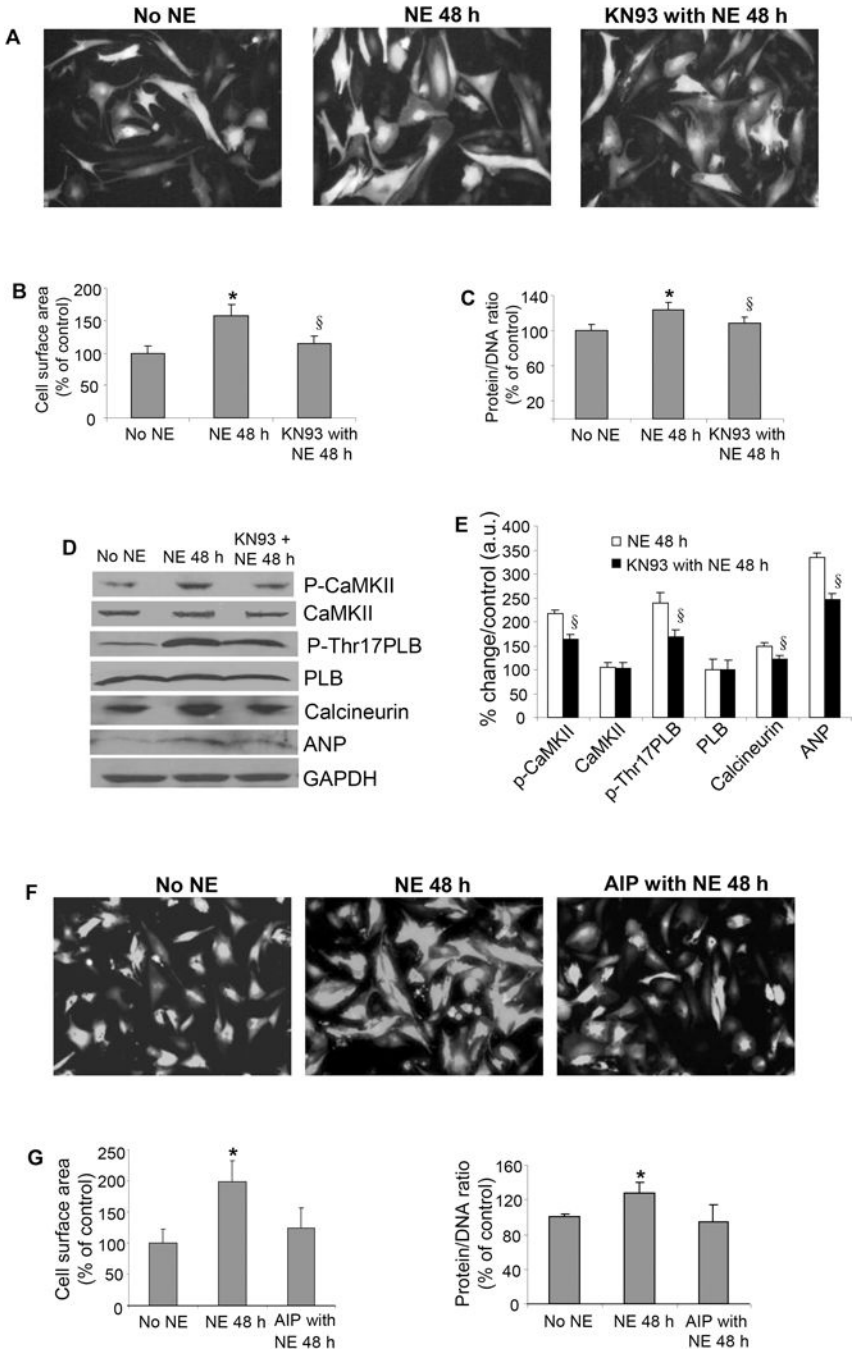

Figure 2. Inhibition of CaMKII attenuates NE-induced hypertrophic response.

Cultured NRVMs were incubated with either 1 μM KN93 or equal amount of DMSO (solvent for KN93) for 2 h and then exposed to 20 μM NE for 48 h at 37 °C. Cell images show that KN93 inhibits NE-induced cell enlargement (**A**). Changes in cell surface area and protein/DNA ratio are summarized in panel **B** and **C**, respectively. Panel **D** shows representative Western blots in NRVMs after exposure to NE for 48 h with and without KN93. Percentage changes in phospho-CaMKII, CaMKII, P-Thr17PLB, PLB, calcineurin and ANP protein levels are summarized in panel **E**. Cell images in panel **F** show inhibition

of NE-induced hypertrophic response by CaMKII inhibitor AIP (10 μM). Cell surface area and protein/DNA ratio in myocytes treated with AIP are summarized in panel **G**. Statistical data are mean values from 3 independent experiments. *: $p < 0.05$, compared to control (No NE). §: $p < 0.05$, compared to NE48h.

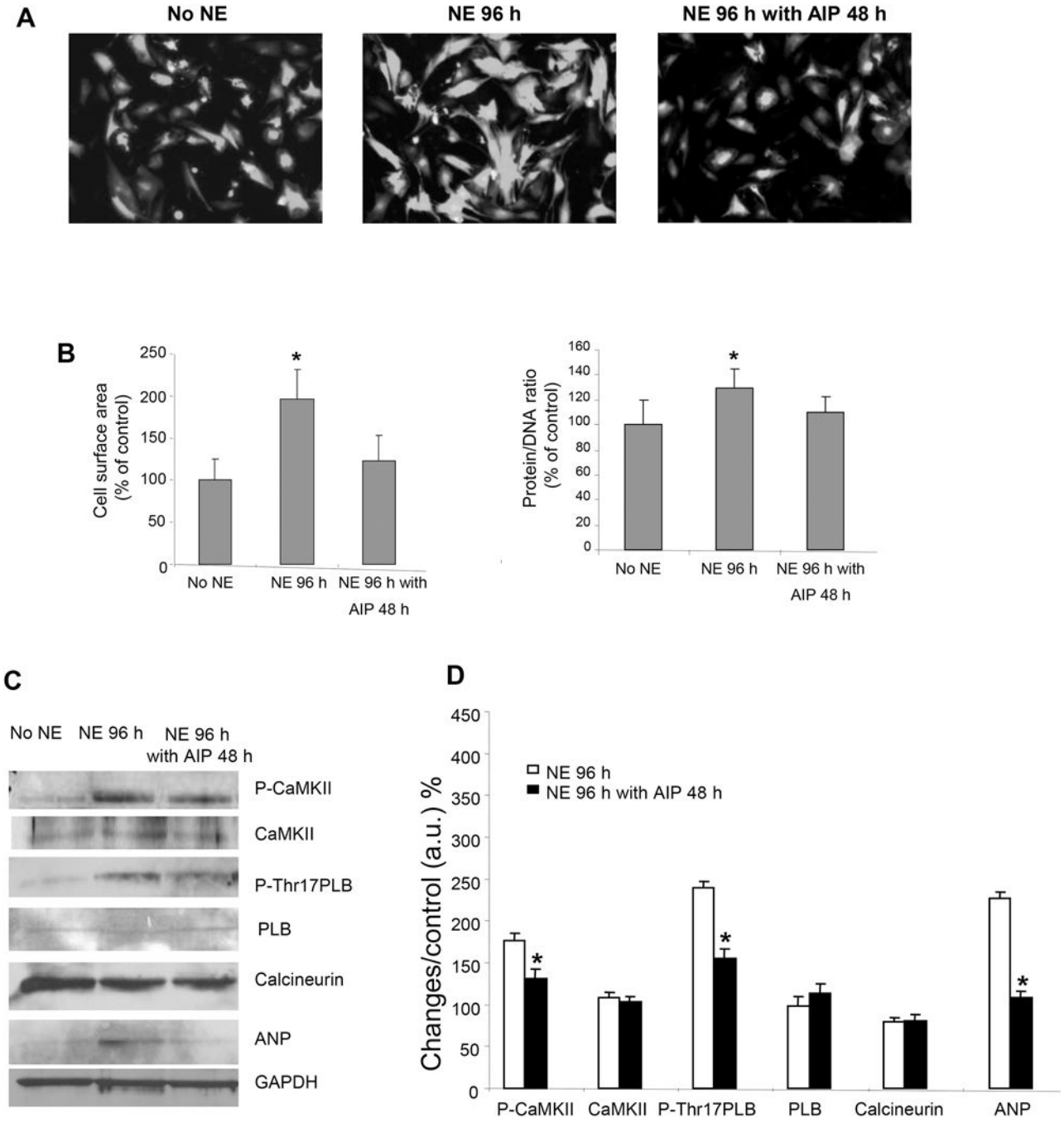

Figure 3. Inhibition of CaMKII reversed NE-induced myocyte hypertrophy.

Cultured NRVMs were incubated with 20 μM NE for 48 h, 10 μM AIP was then added in the culture medium for another 48 h at 37 °C. Cell images in Panel **A** show that AIP reversed NE-induced myocyte enlargement. Panel **B** shows statistical results for the changes in cell surface area and protein/DNA ratio. *: $p < 0.05$, compared to control (No NE). Panel **C** shows representative Western blots and panel **D** shows changes in protein levels of phospho-CaMKII, CaMKII, P-Thr17PLB, PLB, calcineurin, and ANP. Data are mean values from 3 independent experiments. * denote $p < 0.05$, compared to "NE 96 h".

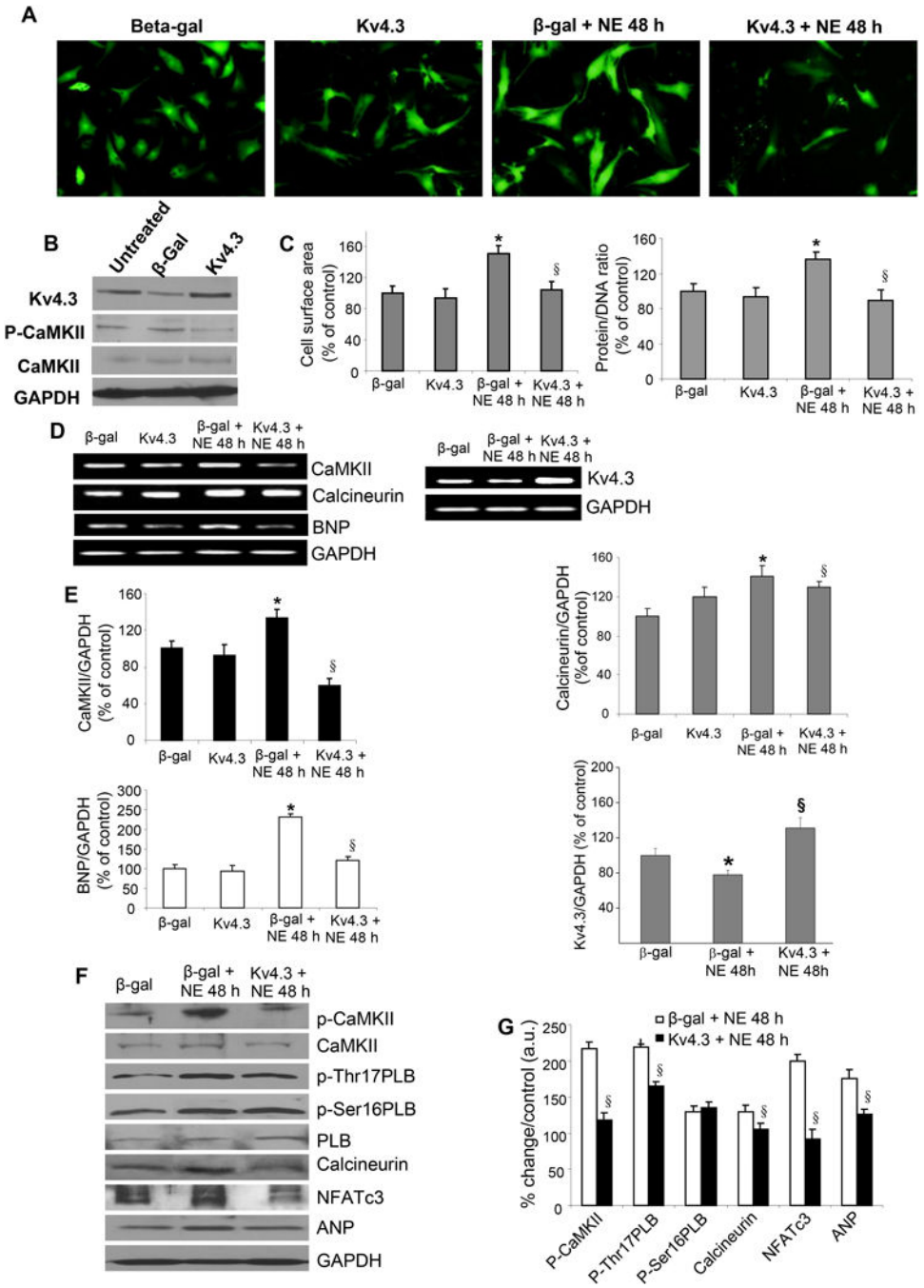

Figure 4. Kv4.3 overexpression blocks NE-induced hypertrophic response by reducing CaMKII and calcineurin activity.

Panel **A**: Cell images show that the hypertrophic response was abrogated in NRVMs transfected with Ad-Kv4.3 but not in NRVMs transfected with Ad-β-gal. Panel **B:** Kv4.3 protein expression was significantly increased in the lysate of NRVMs transfected with Ad-Kv4.3 compared with those from the untreated NRVMs and NRVMs transfected with Ad-β-gal (48h). In addition, NRVMs transfected with Ad-Kv4.3 showed the lowest level of autophosphorylated CaMKII. Panel **C:** The analysis results of cell surface area and

protein/DNA ratio in response to the treatments. Panel **D:** The representative RT-PCR results for mRNA levels of CaMKII, calcineurin, Kv4.3 and BNP, the statistical results are shown in Panel **E**. Representative Western blots are shown in Panel **F**, and the statistical protein levels are summarized in Panel **G**. All statistical data (panel **C**, **E** and **G**) are from 3 independent experiments. * and § denote $p < 0.05$, compared to "Ad-β-gal" and "Ad-β-gal + NE 48 h", respectively.

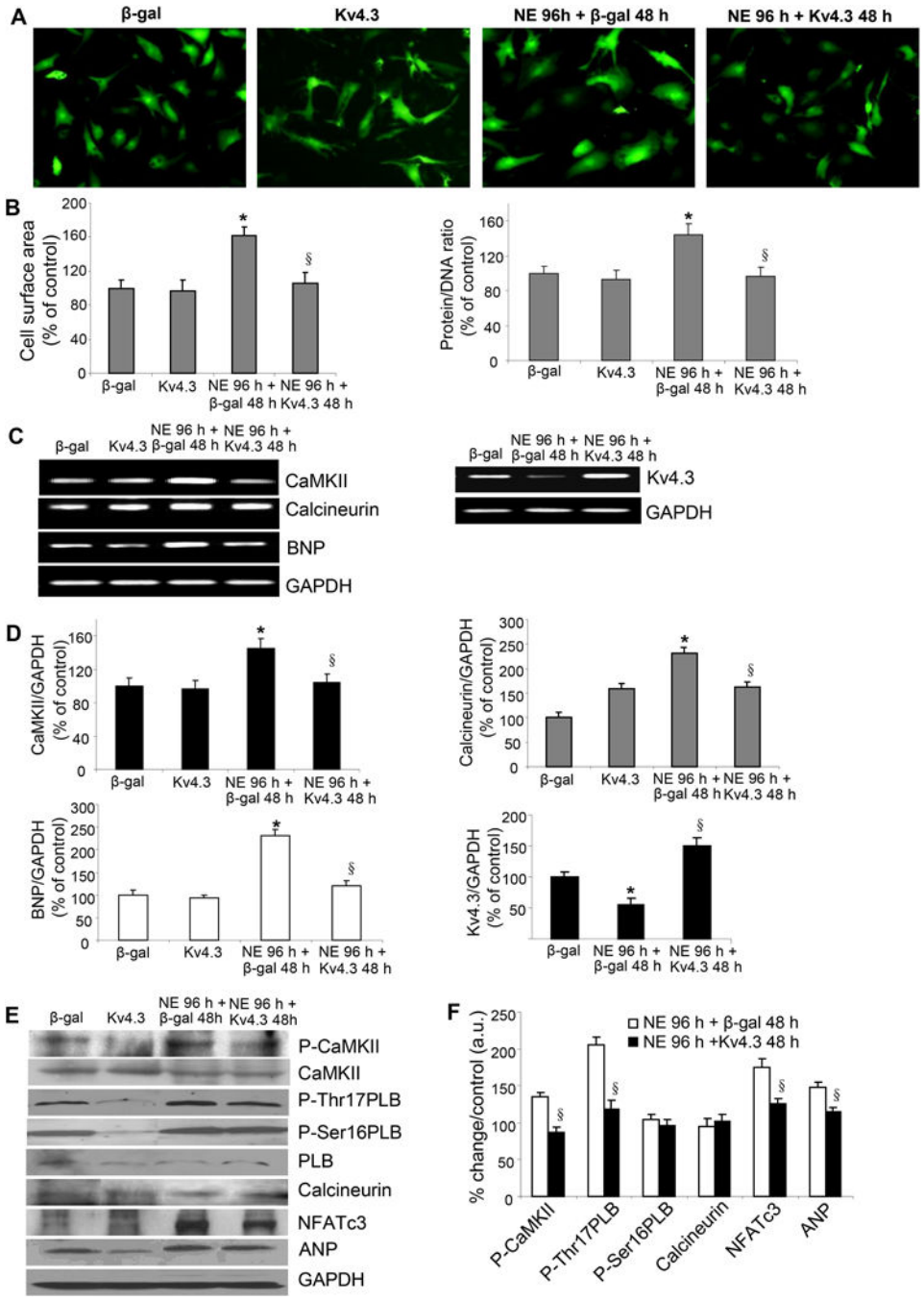

Figure 5. Kv4.3 reverses NE-induced hypertrophy in cultured NRVMs.

Cultured NRVMs were incubated with 20 μM NE for 48 h and then transfected with either Ad-β-gal or Ad-Kv4.3 (at MOI of 200) for another 48 h at 37 °C. Cell images in Panel **A** show that Kv4.3 overexpression reverses NE-induced myocyte hypertrophy. Cell surface area and protein/DNA ratio analysis results are shown in Panel **B**. The representative RT-PCR results for mRNA levels of CaMKII, calcineurin, Kv4.3 and BNP are shown in Panel **C**, and the statistical results are shown in Panel **D**. Representative Western blots are shown in Panel **E**, and the statistical protein levels are shown in Panel **F**. All quantitative data

(panel **B**, **D** and **F**) are from 3 independent experiments. * and § denote $p < 0.05$, compared to “Ad-β-gal” and “Ad-β-gal 48 h + NE 96 h”, respectively.